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13. ABSTRACT *(Maximum 200 Words)*

The difference between metastatic and localized breast cancer are not known. However, genetic instability is a poor prognostic factor in many types of cancer, implying that processes that lead to the gain, loss, or rearrangement of genomic DNA are important in the evolution of cancer. Telomeres are protein-DNA Complexes that cap the ends of linear chromosomes, protecting them from degradation and fusion. In most cells a number of processes lead to the cumulative reduction of telomere length and cell cycle arrest. Some cells, including cancer progenitor cells, are able to up regulate telomerase, the enzyme that adds telomere repeats, and bypass cell cycle arrest. The purpose of this study is to determine if it is possible to differentiate patients with aggressive metastatic breast carcinoma from those with a less aggressive localized disease using telomere DNA content as a prognostic marker. To that end it is important to ascertain what effect telomerase has on telomere DNA content. Here we report that telomere DNA content predicts outcome in breast tumors and in tumor-adjacent normal breast tiddue and that high levels of hTERT mRNA are correlated with known prognostic markers including: tumor grade, S-phase, pliody and metastasis.

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Introduction:

The genetic differences between aggressive metastatic and less aggressive localized breast cancer are not known. However genetic instability is a poor prognostic factor in many types of cancer, including breast cancer, implying that processes that lead to the gain, loss, or rearrangement of genomic DNA are important in the evolution of cancer (Duesberg et al, 1998). Telomeres are protein-DNA complexes that cap the ends of linear chromosomes, protecting them from degradation and fusion (Allsop et al, 1995; Biessmann and Mason, 1992; Blackburn 2000). A number of processes, both stochastic and abrupt, lead to the cumulative reduction of telomere length and ultimately cell cycle arrest or apoptosis (Allsop et al, 1995; Blackburn 2000, Bissemann and Mason, 1992). Some cells, including cancer progenitor cells, are able to up regulate telomerase, the enzyme that adds telomere repeats, and by pass cell cycle arrest. Interestingly, telomerase does not usually lengthen telomeres in cancer cells; instead it maintains them at the length when telomerase is upregulated (Blackburn, 2000; Autexier and Greider, 1996, Blasco et al, 1996).

The purpose of this study was to gain training in breast cancer research by determining if it is possible to differentiate patients with aggressive metastatic breast carcinoma from those with a less aggressive localized disease using telomere DNA content as a prognostic marker. To that end it was important to ascertain what effect telomerase has on telomere DNA content. Elucidating the role these proteins play in telomere length regulation is important if telomere DNA content is to be used as a prognostic tool and to further understanding of the mechanisms that drive carcinogenesis.

Tasks:

The agreed upon tasks to be completed in the statement of work were as follows:

1. Perform a preliminary retrospective investigation of the relationship between telomere DNA content and outcome in breast carcinoma.
2. Determine the relationship between telomere DNA content and c-myc amplification.
3. Determine the relationship between telomere DNA content and hTERT expression in breast carcinoma.

Progress Relative to the Statement of Work:

Task 1: A study population of 62 women diagnosed with breast cancer prior to 1995 was identified in cooperation with the New Mexico Tumor Registry (NMTR). The study population was selected such that approximately half of the women died of metastatic breast cancer, although these women had similar prognostic markers at the time of diagnosis as the control group who survived eight years or greater without signs of disease recurrence. Abstracted patient files were reviewed and available vital statistics were recorded including: age at diagnosis, tumor grade and size, estrogen/progesterone receptor status, treatment and length of survival or date and cause of death. Archival paraffin-embedded tumor was obtained for 48 women. For 34 of these women it was also possible to obtain genetically matched tumor-adjacent normal tissue.

Tissue type and tumor grade were confirmed histologically by Dr Nancy Joste, Department of Pathology, University of New Mexico School of Medicine. DNA was extracted from a total of 96 paraffin-embedded blocks including 48 tumor and 34 tumor-adjacent normal tissues using the

Qiagen Qiamp Tissue Kit (Qiagen). Purified DNA was quantified with a commercial fluorescence-based DNA detection reagent (Picogreen, Molecular Bioprobes, Inc). Telomere DNA contents (TC) were determined using a slot blot-based approach as described (Fordyce et al, in press). Briefly, DNA is denatured, fixed to a nylon membrane and probed with a telomere specific probe end labeled with fluorescein. An antibody conjugated to alkaline phosphatase detects the fluorescein antigen. Addition of the substrate, CDP-Star (Applied Biosystems) produces light. Blots are exposed to film, and the digitized images are quantitated with Nucleotech Gel Expert Software 4.0 (Nucleotech). TC in tumor and tumor-adjacent tissues is expressed as fraction of the placental control run on each blot.

We analyzed TC in a total of 48 breast tumors, comprising two overlapping breast study groups. The first group was composed of paraffin-embedded tumor specimens from 28 women. These tumors were selected so as to have a mixture of positive and negative prognostic markers. For example, tumors were typically estrogen and progesterone receptor-positive, but also large with nodal involvement. Tumors were stratified by median TC. TC was not associated with ethnicity, age at diagnosis, tumor size, tumor grade, nodal involvement or estrogen- or progesterone-receptor status (data not shown). However, as shown in Figure 1A, reduced TC was associated with patients who developed recurrent breast cancer within 84 months of surgery ($p<0.01$). Moreover, Kaplan-Meier analysis demonstrated that telomere DNA content was associated with clinical outcome (Figure 1B, $p=0.01$). The relative risk of breast cancer recurrence was nine times greater in women whose tumors had TC less than the median. To confirm and extend the observed relationship between TC and clinical outcome, an additional 20 randomly selected patients were added to the initial study group. Consistent with our preliminary observation, TC also predicted clinical outcome in the expanded group of patients whose tumors' characteristics were more diverse (data not shown, $p=0.03$.)

In addition to the breast tumors, we analyzed TC in a total of 34 paired tumor-adjacent normal paraffin-embedded breast tissues. Tumor-adjacent normal breast tissues were stratified by median TC. TC of the tumor-adjacent normal tissue was not associated with patients' ethnicity, age at diagnosis, tumor size, tumor grade, nodal involvement or estrogen- or progesterone-receptor status (data not shown). However, remarkably, as shown in Figure 1C, reduced TC was associated with patients who developed recurrent breast cancer within 84 months of surgery ($p=0.005$). Moreover, Kaplan-Meier analysis demonstrated that telomere DNA content in tumor-adjacent normal breast was associated with clinical outcome (Figure 1D, $p=0.001$). *Collectively, these findings demonstrate that TC is an informative prognostic marker in breast tumors and surprisingly in tumor-adjacent normal tissue, and that changes in telomere length may occur early in the neoplastic process, even prior to phenotypic changes.*

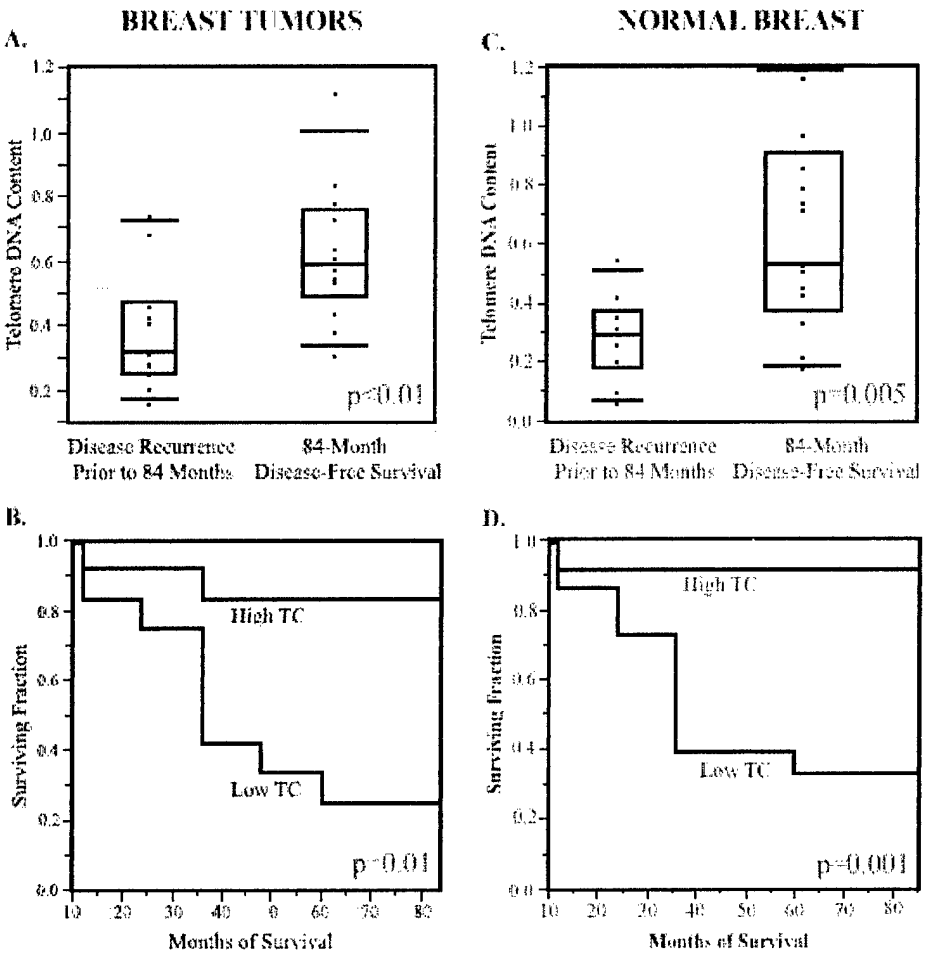
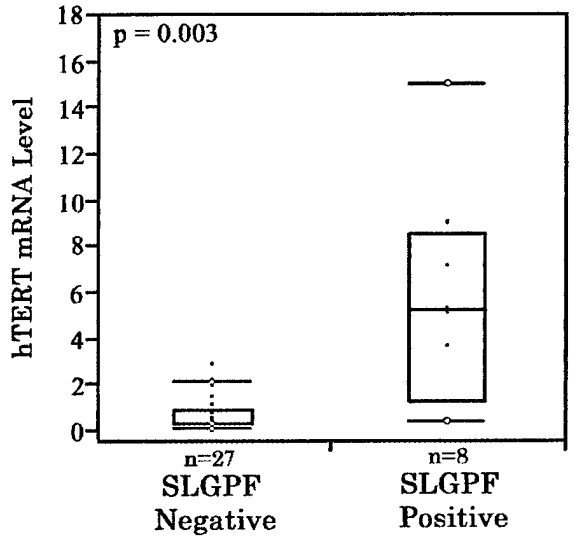


Figure 1: Telomere DNA Content in Tumor and Tumor-Adjacent Normal Breast Tissue. Data from breast tumors are shown in panels A and B, tumor-adjacent, genetically matched normal breast tissues are shown in panels C and D. In panels A and C, breast tissues were divided into two groups, recurrent and disease-free, based on patient outcome 84 months after surgery. P values were calculated by Wilcoxon-Rank Sums analysis. Box plots show patients outcome on the X-axis and TC, as a fraction of the placental control, on the y-axis. In panels B and D breast tissues were divided into groups, high and low, based on median TC. TC greater than or equal to the median is defined as High TC and TC less than the median is defined as Low TC. P values were calculated by Kaplan-Meier analysis. Plots show months of disease-free survival up to 84 months on the X-axis and surviving fraction on the y-axis.

Task 2: Initially it was proposed that the levels of c-myc in tumor samples would be analyzed by quantitative RT-PCR. Since this proposal was submitted it has been possible to utilize Real Time PCR technology. Real time PCR is more sensitive and accurate than quantitative RT-PCR. It was not possible to purify RNA, the starting component of real time PCR, from paraffin-embedded archival tissue; therefore a second study population was identified. Tissue from this second group was stored frozen and was therefore a good source of RNA. The second study population consisted of 50 women diagnosed with breast cancer prior to 1993. Tumor tissues were obtained from mastectomy, lumpectomy or biopsy. Because it was necessary to use only frozen tissue, the members in the second study population were not matched for age, specific type of breast tumor, tumor grade, age at diagnosis estrogen or progesterone receptor status or survival. Dr. Nancy Joste confirmed tumor grade and tissue type.

It was possible to obtain RNA and DNA from 37 of the frozen breast tissues. RNA was quantitated using absorbance spectra. DNA was quantitated as described previously. Although the original statement of work included an analysis of c-myc mRNA levels using real time RT-PCR, this task has not been performed. In 2000, Bieche and coworkers published a paper in which they examined the relationship between hTERT and c-myc mRNA levels and several other prognostic markers in breast cancer. They demonstrated that c-myc mRNA over expression was correlated highest levels of hTERT mRNA expression. As described in Task 3, we found that high hTERT mRNA levels were associated with several tumor markers and was consistent with the data reported by Bieche (Bieche et al, 2000). Thus, analysis of c-myc was not performed.

Task 3: The second study group has been used as a source of RNA to measure the levels of hTERT expression in breast tumors. Typically, the tumors were large (>2 cm), high grade (2 or 3), aneuploid (73%), metastatic (56%) infiltrating ductal carcinomas (74%). Levels of hTERT mRNA were measured by real-time RT-PCR. Levels of hTERT ranged from 0.00 to 22.42 arbitrary units, and differed by 750-fold. Only the smallest in the study (0.8 cm in largest dimension) did not express detectable hTERT mRNA. There was no apparent relationship between hTERT mRNA level and telomere DNA content or the proportion of contaminating normal cells.



The hTERT mRNA levels in the 37 tumors, grouped by the characteristics described in Table 2, were analyzed using Wilcoxon rank sum analysis. Levels of hTERT mRNA were greater in tumors that were large (p=0.012), mitotically active (p=0.024) and high grade (p=0.033). Most importantly, as shown in Figure 2, the tumors expressing the highest levels of hTERT mRNA also possessed all five negative prognostic markers examined in this study; large, node-positive, high grade, aneuploid and mitotically active (p=0.003).

Figure 2: hTERT mRNA Levels in Breast Tumors. Tumors which possessed all five negative characteristics: large size, nodal metastasis, high grade (III), high S-Phase fraction and aneuploid (SLGPF) also expressed the highest levels of hTERT mRNA.

Key Research Accomplishments:

- Identified a study population, half of whom survived greater than eight years without disease recurrence and half of whom died from metastatic breast cancer. This study population had similar prognostic markers at the time of diagnosis.
- Purified and quantitated genomic DNA from tumor normal pairs of the first study population.
- Telomere DNA content was analyzed in 48 tumors and 34 tumor-adjacent normal tissues.

- Identified a second study population, whose tissues have been frozen, thus preserving the integrity of tumor RNA.
- Developed real time RT-PCR assay for hTERT, the catalytic subunit of telomerase.
- Purified and quantitated genomic DNA and RNA from tumor tissues from the second study population.
- Completed analysis of 37 tumors for levels of hTERT expression using real time RT-PCR and for telomere DNA content using the slot blot assay.

Reportable Outcomes:

- Two databases have been produced that contain anonymous patient histories, including age at diagnosis, treatments, tumor grade, estrogen and progesterone receptor status, tumor size, length of disease free survival or date and cause of death and diagnosis.
- DNA and RNA banks from tumor and normal breast tissues have been produced.
- Tissue culture cell lines, which contain the hTERT and TBP genes that are used as controls for real time PCR have been produced.
- These data were presented at the FASEB meeting in New Orleans in April of 2002.
- Manuscript: "Chemiluminescent Measurement of Telomere DNA Content in Biopsies" is in press in *Biotechniques* (Appendix).
- Patent application is pending for Chemiluminescent Measurement of Telomere DNA Content in Biopsies.
- Manuscript: "Telomere DNA Content Predicts Clinical Outcome in Breast and Prostate Cancer" submitted to the *Journal of the National Cancer Institute* (Appendix).
- Manuscript: "hTERT mRNA Levels are Associated with Poor Prognostic Markers in Breast Cancer" in preparation.
- Colleen Fordyce has been supported by this training grant and earned her Ph.D. in Biomedical Sciences in May of 2002. She has gained expertise in database development, DNA and RNA purification from archival tissues, slot and southern blots and real time RT-PCR methodologies.

Conclusions:

- Telomere DNA content is a novel and independent prognostic marker in breast tumors and tumor-adjacent normal tissue. These data imply that changes in telomere length may occur early in the neoplastic process, even prior to phenotypic changes.
- hTERT levels correlate with several known prognostic markers including: tumor grade, ploidy, % of cells in S-phase, and metastasis effectively identifying the subset of tumors with the worst prognostic markers. Therefore hTERT may be a "meta-prognostic marker" and could be used to identify the most aggressive tumors.

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Molecular Diagnostic Techniques

Short Technical Report

Chemiluminescent Measurement of Telomere DNA Content in Biopsies

BioTechniques 33:___ (July 2002)

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ABSTRACT

Telomeres are nucleoprotein complexes that protect the ends of chromosomes from fusion and degradation. They are typically shorter in tumor cells than in paired normal cells, and shorter telomeres are associated with poor outcome in cancer. We previously described a slot blot–based method for measuring telomere DNA content, a proxy for telomere length. Although this method represented an improvement over existing methods, its 3-ng limit of sensitivity was insufficient for use with biopsy or other scant tissues. Here we describe a chemiluminescent slot blot assay for telomere DNA content that has the sensitivity required for use with biopsy material. The results obtained with DNA derived from human placental, HeLa, human peripheral blood lymphocytes, sham-needle core prostate biopsies, and archival prostatectomy tissues demonstrated that telomere DNA content can be reliably and reproducibly measured in 5 ng, and sometimes as little as 2 ng, genomic DNA. Sham-needle core prostate biopsy and prostatectomy specimens processed in parallel produced comparable results. The contribution of truncated telomeres in admixtures containing as much as 75% normal placental DNA could be established. We also demonstrated that the treatment of tissue with formalin before DNA purification does not decrease the efficacy of the assay.

INTRODUCTION

Telomeres are nucleoprotein complexes that protect the ends of chromosomes from fusion and degradation (1,3,6,11,12). Because telomeres are shortened each time a cell divides (1,3, 11,15), tumor cells typically have shorter telomeres than paired normal cells (1,3,6,16). Reduced telomere DNA content (or length) has been associated with aneuploidy, metastasis, grade, disease recurrence, and survival (2,4,5,7–10,13,14,17). To validate the potential prognostic significance of telomere DNA content, it is necessary to measure telomere DNA content in relevant clinical specimens, particularly in biopsies.

We previously described a slot blot method for measuring telomere DNA content, a proxy for telomere length (5). In contrast to the commonly used Southern blotting method of measuring terminal telomere restriction fragments (1,10,13,16), the slot blot assay required less than 0.1 µg genomic DNA and was insensitive to DNA breakage (5). Nevertheless, it lacked the sensitivity required for use with biopsy specimens and microdissected or other very scant tissues took 5–8 days to complete and relied on hybridization of a second centromere-specific oligonucleotide to normalize for DNA quantity/load and reactivity. In addition to these limitations, the effects of formalin fixation and normal cell contamination in tumors were unknown. These factors limit the potential clinical utility of the slot blot assay for use with biopsy specimens.

To address these limitations, we developed a new chemiluminescent assay for measuring telomere DNA content that is both more sensitive and faster than previous methods. We also tested the assay's suitability for use with DNA purified from formalin-fixed tissue and admixtures of normal and tumor cells.

MATERIALS AND METHODS

DNA Isolation

DNA was purified from human placental tissue and cultured HeLa cells as previously described (5). Waste frozen human prostate tissue was obtained from the University of New Mexico Solid Tumor Facility. Sham-needle core biopsies were produced from the frozen prostate tissue using a 2-cm-long, 18-gauge needle core. Pieces (0.2–0.4 g) of human placental tissue were submerged in formalin (Sigma, St. Louis, MO, USA) for 0, 15, 30, 60, 120, 240, and 480 min at room temperature. The tissue was washed twice in 30 mL PBS (Sigma) to remove any residual formalin before DNA purification. DNA was purified from sham-needle core biopsies and formalin-treated placenta using the DNAeasy Kit and the manufacturer's protocol (Qiagen, Valencia, CA, USA). The average yield of DNA from a 2-cm-long, 18-gauge frozen needle core was 150 ng ($n = 9$; 159 ng SD). The DNA yield was not affected by formalin fixation. Archival paraffin-embedded prostatectomy tissue was obtained from the New Mexico Tumor Registry as approved by the Human Research Review Committee of the University of New Mexico). DNA was purified from archival paraffin-embedded tissue as described above.

Quantification of DNA

Placenta, HeLa, sham-needle core biopsy, and paraffin-embedded prostatectomy DNAs were quantitated with the fluorescent dye, PicoGreen® (Molecular Probes, Eugene, OR, USA), using a commercially available human genomic DNA purified from peripheral blood lymphocytes (Promega, Madison, WI, USA) as a standardized control. PicoGreen was added to both the standard and purified DNAs and excited at 480 nm, according to the manufacturer's instructions. Fluorescence emission intensities were measured at 520 nm using a Luminescence Spectrometer LS50 (Perkin Elmer Life Sciences, Gaithersburg, MD, USA). Concentrations of the DNA samples were calculated from the equation describing the best-fit line generated with the standardized control DNA.

Preparation and Hybridization of Slot Blots

Slot blots were prepared as previously described (5), with the following modifications. DNA was applied to Tropilon Plus™ membranes (Tropix, Bedford, MA, USA), air-dried, and cross-linked with 1200 mJ (UVP, San Gabriel, CA, USA). Following cross-linking, each blot was submerged in 0.25 *M* sodium phosphate buffer, pH 7.2. Each blot was prehybrized in a 200-mL glass hybridization bottle (Belleo Glass, Vineland, NJ, USA) for 1 h at 60°C in 50 mL hybridization buffer (7% SDS, 0.25 *M* sodium phosphate buffer, pH 7.2, 0.001 M EDTA). Following prehybridization, the buffer was replaced with 50 mL fresh hybridization solution containing 300 pmol telomere-specific fluorescein 3′-end-labeled probe [5′-TTAGGG-3′]$_4$ (IDT, Coralville, IA, USA). Each blot was hybridized at 60°C for a minimum of 12 h and a maximum of 16 h. Following hybridization, the blot was washed twice at room temperature for 5 min in 30 mL 2× SSC and 1% SDS. The initial wash was followed by two high-temperature washes at 60°C for 15 min in 30 mL preheated 1× SSC and 1% SDS. Finally, the blot was washed twice at room temperature for 5 min in 30 mL 1× SSC. All washes were performed in glass hybridization bottles with constant agitation in an AutoBlot Hybridization Oven (Belleo Glass).

Detection and Quantification of Telomere DNA

After hybridization, telomere DNA was quantitated using the Southern Star™ Chemiluminescent Kit, as described by the supplier (Tropix). The blots were equilibrated for 5 min in two successive 20-mL washes of blocking buffer [PBS, 2% I-block reagent (Tropix), and 0.1% Tween® 20 (Sigma)]. Each blot was then incubated separately for 45 min in 30 mL blocking buffer at room temperature with constant agitation. The blocking buffer was discarded and replaced with 50 mL blocking buffer containing 2 µL alkaline phosphatase-conjugated, anti-fluorescein antibody (Tropix). The blot was incubated for 30 min at room temperature with constant agitation, washed in 30 mL fresh blocking buffer, and then washed three times for 5 min at room temperature in 30 mL wash buffer (PBS and 0.1% Tween 20) with constant agitation.

The pH of the blot was optimized for alkaline phosphatase activity by incubating the blot twice for two min in 20 mL 1× assay buffer (Tropix). The surface of the blot was completely covered with approximately 4 mL CDP-Star® Chemiluminescent Substrate (Tropix) and incubated for 5 min at room temperature (Figure 1A). Following incubation, CDP-Star was wicked away, and the blot was sealed in a plastic bag and exposed to Hyperfilm (Amersham Biosciences, Little Chalfont, UK) for 2 or 5 min.

The films were developed using a medical film processor (model QX-70; Konica) and scanned using a ScanJet ADF (Hewlett Packard). The intensity of the telomere hybridization signal was determined from the digitized images with the Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA, USA). Telomere content was determined by comparing the slope of the equation generated from DNA input versus the relative signal from the placental standard to the samples at known concentration (Figure 1B).

RESULTS AND DISCUSSION

Sensitivity and Specificity

There was a linear relationship (R^2 = 0.98-99) between the telomere DNA content and inputs of 5–120 ng in human genomic DNAs purified from placenta, HeLa cells, and peripheral blood lymphocytes. Telomere contents in the placenta and the peripheral blood lymphocyte DNAs, representative of normal somatic cells, were nearly identical (slopes = 923 vs. 942, respectively). In contrast, the telomere content in HeLa DNA, representative of tumor cells, was significantly decreased (slope = 353) (Figure 1B). Telomere DNA contents in genomic DNA isolated from sham-needle core biopsies ranged 48%–100% of the placental control (Figure 1C). There was no signal when equivalent amounts of a nonspecific plasmid DNA (pGEM®-easy vector; Promega) were substituted for genomic DNA in the assay (data not shown).

Telomere Contents in Admixtures

Tumor and biopsy specimens typically contain mixtures of normal cells and tumor cells, whose telomeres differ in length. Thus, the observed telomere content of such a specimen reflects both the telomere content and relative proportions of normal and tumor cells. The observed telomere content (TC_{obs}) can be expressed quantitatively by the following equation:

$$TC_{obs} = (TC_N)(\%N) + (TC_T)(\%T) \quad \text{[Eq. 1]}$$

where TC_N and TC_T are the telomere contents in normal and tumor cells, respectively, and %N and %T are the percentages of normal and tumor cells in the specimen, respectively. Ideally, the telomere content of the tumor cells in the mixed samples could be calculated with the following equation:

$$(TC_T) = [TC_{obs} - (TC_N)(\%N)]/(\%T) \quad \text{[Eq. 2]}$$

where TC_{obs}, TC_N, and %T (defined by histopathological examination) are known. To validate this approach, telomere contents were measured in mixtures containing defined propor-

tions of HeLa cell DNA, mimicking tumor cells, and placental DNA, mimicking normal cells (Figure 2). There was a linear and predictable relationship between the telomere content and the relative proportions of HeLa and placenta DNA in each of the admixtures. Identical results were obtained when telomere contents were assayed in defined admixtures containing 30, 40, 50, or 60 ng total genomic DNA (data not shown). Thus, the contributions of the truncated HeLa cell telomeres in admixtures containing as much as 75% normal placental DNA can be defined.

Telomere DNA Content in Formalin-Fixed Tissue

It is not uncommon for surgical and biopsy specimens to be stored in formalin for as long as 8 h before embedding in paraffin. To determine whether formalin fixation affected the reliability of the telomere DNA content assay, human placenta was submerged in formalin for up to 8 h at room temperature before DNA purification, as shown in Figure 3. Telomere DNA contents measured in genomic DNA isolated from tissue treated with formalin were not different from the untreated control (slopes = 768 and 804, respectively). Telomere DNA content could also be assayed with DNA purified from paraffin-embedded archival prostatectomy specimens (Figure 1A).

CONCLUSION

Although the reported associations between reduced telomere DNA content and several prognostic markers of cancer are provocative (2,4,5,7–10, 13,14,17), prospective investigations with biopsy materials are necessary to establish the clinical relevance of telomere DNA content. Such investigations have not been possible with existing methods. We have developed a

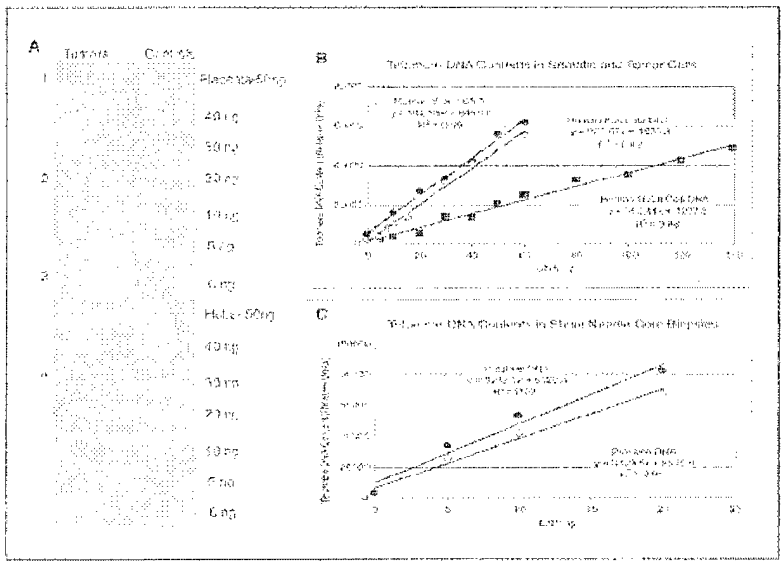

Figure 1. Sensitivity of telomere DNA content assay. (A) Representative slot blot showing placental, HeLa, and five archival paraffin-embedded prostate tumor samples. Placenta and HeLa DNA concentrations range 0–50 ng, and tumor DNAs were analyzed in triplicate (one replicate for sample 5 is not shown) at 40 ng. (B) Representative data demonstrating differential telomere DNA contents in normal somatic (human blood lymphocytes and placenta) and tumor (HeLa) cells. The slot blot was prepared and analyzed as described in the Materials and Methods section. (C) Representative data demonstrating telomere DNA contents in sham-needle core biopsies. Slopes, x-intercepts, and correlation coefficients were calculated by linear regression.

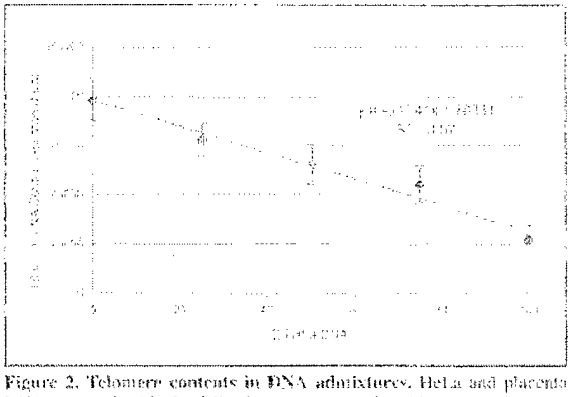

Figure 2. Telomere contents in DNA admixtures. HeLa and placenta DNAs were mixed in the following proportions: 0%, 25%, 50%, 75%, and 100% HeLa. Data shown represents telomere DNA contents measured in 60 ng total genomic DNA analyzed in triplicate. The regression line and correlation coefficient were calculated as in Figure 1. The error bars represent standard deviation of the means.

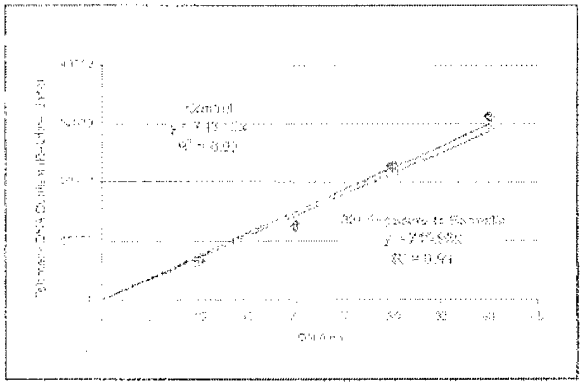

Figure 3. Effect of formalin exposure on telomere content assay. Human placenta was placed in formalin for 0–8 h at room temperature. Telomere DNA contents were measured in DNA that was purified following tissue treatment with formalin. Identical results were obtained from all time points, and data from 0–8 h are shown. See Figure 1 legend for additional details.

chemiluminescent assay for telomere DNA content that is particularly well suited for the analysis of biopsy or microdissected specimens. The assay, including DNA purification and quantification, can be completed in two days and requires as little as 2–5 ug genomic DNA, the equivalent of 285–715 diploid cells. This technique is at least 10 times more sensitive and five times faster than existing methods. In addition, the assay is unaffected by standard formalin treatment and is informative for tumor tissues that contain up to 75% normal cells.

It is important to emphasize that telomere DNA contents determined by this method reflect the average telomere content in cells comprising the sample. It does not provide information about the variability of the telomere DNA content on chromosomes within or among individual cells. However, with the assay's improved sensitivity, telomere DNA content could be measured in microdissected or flow-sorted tumor cell subpopulations.

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We are indebted to our colleagues, Dr. Nancy Joste from the University of New Mexico Department of Pathology and Dr. Lida Crooks from the Albuquerque Regional Administration of Veterans Affairs Medical Center Department of Pathology (Albuquerque, NM, USA) for their invaluable assistance.

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Telomere DNA Content Predicts Clinical Outcome in Breast and Prostate Cancer

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ABSTRACT

Background: Telomeres are protein-DNA complexes that stabilize the ends of chromosomes. Genomic instability, resulting from telomere shortening, creates phenotypic variability that may facilitate the progression to a metastatic phenotype. Thus, we hypothesized that telomere DNA content would predict clinical outcome in breast and prostate cancer.

Methods: Telomere DNA content was measured with a slot-blot based assay developed in our laboratory. Telomere DNA content was analyzed in 48 breast and 51 prostate tumors identified by the New Mexico Tumor Registry. Patient histories included 84-months of follow-up, ethnicity, age at diagnosis, tumor grade or Gleason sum score, nodal involvement and several other tumor markers. An initial breast study was composed of 28 matched breast tumors from patients whose prognoses were ambiguous at diagnosis. This group was expanded with 20 randomly selected breast cancer patients. Statistical analysis was two-sided, used a 95% confidence interval, and was performed with the Wilcoxon Rank sums and Kaplan-Meier tests.

Results: In the initial breast study population, reduced telomere DNA content was associated with disease recurrence ($p<0.01$) and poor clinical outcome ($p=0.01$). These results were extended in the expanded breast study; reduced telomere DNA content was again associated with poor clinical outcome ($p=0.03$). In the prostate study, reduced telomere DNA content was also associated with disease recurrence ($p=0.01$) and poor clinical outcome ($p=0.04$). Telomere DNA content was not associated with any of several tumor markers in either breast or prostate cancer.

Conclusions: Telomere DNA content has potential as an informative, independent prognostic marker in breast and prostate cancer.

INTRODUCTION

Currently available prognostic markers often fail to identify patients with lethal, metastatic tumors. Nodal involvement and either high tumor grade or moderate to high Gleason sum scores are currently the most informative prognostic markers for breast and prostate cancers, respectively (*1-5*). However, these markers often fail to discriminate between patients who will have favorable and poor clinical outcomes (*1,3,5-9*). Accordingly, many patients diagnosed with breast or prostate cancer receive aggressive therapies that may, in fact, be unnecessary. Thus, it is important to develop new prognostic markers that more accurately predict disease recurrence and survival. Recent reports demonstrate the feasibility of using microarray technologies to produce gene-expression signatures of aggressive tumors (*10,11*). However, the costs and complex data output of this technology presently precludes its use in most clinical laboratories. Alternatively, we have developed a simple, inexpensive assay for telomere DNA content (a proxy for telomere length). Here, we show that this assay can be used to predict clinical outcome in breast and prostate cancer.

Telomeres are specialized protein-nucleic acid structures located at the ends of eucaryotic chromosomes (*12*). Shortened telomeres are associated with genomic instability, including dicentric chromosome formation, chromosome translocation and loss of heterozygosity (*13-16*). These data demonstrate that telomeres play a critical role in protecting and stabilizing chromosome ends (*17-19*). Because of incomplete replication of DNA on the leading strand of the replication fork, telomeric DNA is lost each time a cell divides. Thus, cells with a higher proliferative history, like tumor cells, typically have shorter telomeres and are more likely to be genetically unstable (*12,20,21*). Therefore, we hypothesized that the telomere DNA in a tumor would presage a tumor's phenotypic variability and, hence, metastatic potential.

We have previously described a slot blot assay for telomere DNA content. Telomere DNA content is directly proportional to telomere length measured by Southern blotting, and thus serves as a proxy for telomere length (*22*). However, in contrast to Southern blotting (*23,24*), the slot blot assay requires less than 100 ng of genomic DNA and is insensitive to DNA breakage, making it ideal for retrospective studies of archival tissues (*22*). Moreover, we have shown that telomere DNA content measured in frozen tissues and formalin-fixed, paraffin-embedded tissues are comparable (*22*),(*25*). Using this assay, we previously reported that reduced telomere content was associated with aneuploidy and metastasis in breast cancer and with disease recurrence and survival in an initial prostate cancer study (*26*). However, the composition and small size of each cohort limited the significance that could be ascribed to these observations. To investigate the prognostic potential of telomere DNA content more thoroughly, we measured telomere DNA content in two distinct study populations comprised of 48 women with breast tumors and 51 men with prostate tumors.

MATERIALS AND METHODS

Study Populations The New Mexico Tumor Registry (NMTR) database links anonymous patient histories to archival cancer specimens through pathology reference numbers and dates of procedures as approved by the University of New Mexico Human Research Review Committee in accordance with all federal guidelines. A retrospective examination of anonymous records from the NMTR database identified a total of 48 women diagnosed with breast cancer, comprising two overlapping study groups. The first study group was composed of 28 women diagnosed with infiltrating breast cancer prior to 1994. Patients were carefully selected such that their tumors possessed both positive and negative prognostic markers, and as such, their possible outcome was ambiguous at diagnosis. Of the 28 women, 13 either died from or had recurrent breast cancer. Archival paraffin-embedded tumor tissues were obtained for each woman.

An expanded breast study group was composed of 48 women diagnosed with breast cancer prior to 1996, including the 28 women in the first group. No selection criteria, other than availability of frozen or paraffin-embedded tissue, were applied to the remaining 20 women in this group. Patient histories were obtained for all 48 women. Evaluable data typically included ethnicity, age at diagnosis, lymph node metastasis, tumor size, tumor grade and duration of disease free survival or date and cause of death. In approximately one-third of the tumors, estrogen and progesterone receptor status was also available. Nearly all of the women in the study population received one of several adjuvant therapies.

A prostate cancer study group was composed of 51 men diagnosed prior to 1996. Of the 51 men, 21 either died from or had recurrent prostate cancer. Typically, men were treated with prostatectomy and did not receive additional treatments unless or until there were signs of disease recurrence, such as rising PSA (prostate specific antigen). Patient histories included ethnicity, age at diagnosis, Gleason score, lymph node and seminal vesicle metastasis, duration of disease-free survival, and date and cause of death.

Histological Review Tumor tissues were derived from excisional biopsy obtained at diagnosis, partial or full mastectomy or prostatectomy. Both paraffin-embedded and frozen tumor tissue sections were stained with hematoxylin and eosin and were examined microscopically to determine the fraction of normal and tumor cells within each tissue section. Paraffin blocks and frozen tissue sections containing the highest proportion of tumor cells were used for the study.

DNA Isolation DNA was purified from either paraffin-embedded or frozen archival tissue. DNA was extracted from twelve, serial 25 μm-thick sections of paraffin-embedded tissue using the Qiamp Tissue Kit (Qiagen, Valencia CA) with the following modifications to the manufacture's protocol. Tissue sections were deparaffinized in octane and washed in ethanol as described previously (Fordyce, Heaphy and Griffith, in press). Frozen tissues were cut into small pieces using a sterile scalpel. DNA was extracted from frozen tissue using the Qiagen DNAeasy Kit following the manufacture's protocol. Placenta and HeLa DNA controls, with terminal restriction fragments (TRF) lengths of 10.1 and 5.4 KB, were purified as described (*22*).

Quantification of DNA Tumor DNAs were quantitated with the fluorescent dye, PicoGreen (Molecular Probes, Eugene, OR) following the manufacturer's protocol.

Slot Blots Slot blots were prepared as described (Fordyce, et al, in press). Briefly, DNA was denatured, neutralized, loaded and fixed to Tropilon-Plus^{TM} membranes (Applied Biosystems, Foster City, CA). A telomere specific oligonucleotide end-labeled with fluorescein, $(5'TTAGGG3')_4$-FAM, was purchased from IDT (Coralville, IA). Following hybridization, the blots were washed to remove non-hybridizing oligonucleotides. Telomere-specific oligonucleotides were detected by an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when a substrate, CDP-Star, is incubated with the blot (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized. The intensity of the telomere hybridization signal was determined from the digitized images with Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). Telomere DNA content was expressed as a percentage of the average chemiluminescent signal of three replicate tumor DNAs, analyzed in duplicate or triplicate, compared to the value of the placental standard at the same genomic DNA concentration. (In 15 of the 51 prostate tumors, telomere DNA content was measured using an earlier version of this assay (*22*)).

Statistical Analysis Bias in the data analyses was minimized by stratifying tumors according to the median telomere DNA content of each study group. Telomere DNA content equal to or greater than the median was considered "High" and less than the median was considered "Low". Patients were identified retrospectively using their date of surgical procedure as the start date of the study and were followed for 84 months or until death. Except for survival analysis, which was preformed with the Kaplan-Meier test, all statistical tests were two-sided Wilcoxon Rank Sums tests for which the α level was set at 0.05. Jmp In software 3.0 (SAS Institute) was used for all statistical analysis.

RESULTS

Telomere DNA Content and Breast Cancer: DNA was purified and telomere DNA content was analyzed by slot-blot in a total of 48 breast tumors, comprising two overlapping breast study groups. The first group was composed of paraffin-embedded tumor specimens from 28 women. Thirteen of these women developed recurrent breast cancer (including 12 who died from breast cancer) within 84 months of surgery. The remaining 15 women were disease-free for at least 84 months. These tumors were selected so as to have a mixture of positive and negative prognostic markers. For example, tumors were typically estrogen and progesterone receptor -positive, but also large with nodal involvement (Table 1). Telomere DNA content was not associated with ethnicity, age at diagnosis, tumor size, tumor grade, nodal involvement or estrogen- or progesterone-receptor status (data not shown). However, as shown in Figure 1a, reduced telomere DNA content was associated with patients who developed recurrent breast cancer within 84 months of surgery ($p<0.01$). Moreover, when tumors were stratified by median telomere DNA content, Kaplan-Meier analysis demonstrated that telomere DNA content was associated with clinical outcome (Fig. 1b, $p=0.01$). The relative risk of breast cancer recurrence was nine times greater in women whose tumors had telomere DNA content less than the median.

To confirm and extend the observed relationship between telomere DNA content and clinical outcome, an additional 20 randomly selected patients were added to the initial study group. Seven of these 20 women developed recurrent breast cancer (including six who died of breast cancer) within 84 months of surgery. The characteristics of the 48 tumor specimens in the expanded study group are shown in Table 1. Consistent with our initial observation, telomere DNA content also predicted clinical outcome in the expanded group of patients (Fig. 2, p=0.03).

Telomere DNA Content and Prostate Cancer: Using a carefully matched, limited prostate cancer study group (analogous to the initial breast cancer study group) we previously demonstrated that reduced telomere DNA content was also associated with disease recurrence and death (*26*). This result, in combination with investigations of breast cancer described above, implies that telomere DNA content has prognostic significance in cancers with both a variety of markers and different tissues of origin. To evaluate this possibility, telomere DNA content was analyzed in paraffin-embedded prostate tumors from 51 randomly selected men. Twenty-one of these men developed recurrent prostate cancer (including 13 who died from prostate cancer) within 84 months of surgery. The remaining 30 men remained disease-free for at least 84 months. The characteristics of the prostate study group are summarized in Table 2. Similarly, there was no association between telomere DNA content and ethnicity, age at diagnosis, Gleason sum score or lymph node or seminal vesicle status (data not shown). However, as shown in Figure 3a, reduced telomere DNA content was again associated with patients who either died from, or developed recurrent cancer within 84 months of surgery (p=0.01). Moreover, when tumors were stratified by median telomere DNA content, Kaplan-Meier analysis demonstrated yet again, that telomere DNA content was associated with clinical outcome (Fig. 3b, p=0.04).

DISCUSSION

Currently available prognostic markers often fail to accurately identify patients with the potential for lethal, metastatic tumors. Accordingly, cancer patients often receive empiric, aggressive therapies that, in fact, may not be necessary. Thus, there is a pressing need to identify more informative prognostic markers. To that end, we have evaluated the hypothesis that tumors with the shortest telomeres have the most aggressive phenotypes, which is suggested by our previous studies (*13,25-28*). This hypothesis is based on the fact that critically shortened telomeres are associated with genomic instability (*13,14,27*), and the assumption that genotypic instability results in phenotypic variability.

Although telomere loss has been associated with disease progression in a number of cancers, there have been few investigations of telomeres and clinical outcome(*29-32*). In 1998, Bechter and coworkers demonstrated that reduced telomeres were associated with decreased survival in patients with B cell chronic lymphocytic leukemia (*33*). Similarly, we showed that telomere loss was associated with reduced survival and increased disease recurrence in a preliminary investigation of prostate cancer (*26*). While these investigations are provocative, they either lacked sufficient sample size or a direct association with patient outcome.

The current investigation was designed to explore the hypothesized relationship between telomere DNA content and clinical outcome in breast and prostate cancer, diseases in which it is often difficult to predict disease recurrence. Here we show, in two independent studies, that

telomere DNA content is associated with clinical outcome. Our investigation of telomere DNA content in breast cancer included two overlapping study groups. The initial group was carefully selected to contain women whose tumor markers made it difficult to differentiate between patients with aggressive, lethal tumors and those with less aggressive disease, i.e. the women with the most problematic prognoses. In this group, telomere DNA content predicted death from breast cancer or disease recurrence ($p=0.01$). To ensure that this result was not an artifact of the study population, the analysis was repeated on an overlapping, less homogeneous group. This group, which was nearly double the size of the initial population, was formed by the addition of randomly selected patients. In the expanded study group, telomere DNA content also predicted death from breast cancer or disease recurrence ($p=0.03$).

In a second, independent study we examined the relationship between telomere DNA content and outcome in prostate cancer. This study was undertaken in part to confirm and extend our previously reported findings that reduced telomere DNA content was associated with death and disease recurrence in a limited prostate study group (*26*). In contrast to the initial prostate study, no selection criteria were applied to the patients. Once again, reduced telomere DNA content predicted death from prostate cancer or disease recurrence ($p=0.04$).

Telomere DNA content was not associated with patients' ethnicity, age at diagnosis, tumor size or nodal involvement in either breast or prostate cancer, nor with estrogen- nor progesterone-receptor status in breast cancer, nor Gleason sum score nor seminal vesicle status in prostate cancer. Thus, telomere DNA content is independent of these existing, prognostic tumor markers. Collectively, these data provide strong support for the hypothesis that telomere DNA content is an informative, independent and novel prognostic marker for breast and prostate cancer.

Several characteristics of the telomere DNA content assay make it well-suited for clinical use: it requires very small quantities of DNA that can be isolated from fresh, frozen or paraffin-embedded tissue; the results are not affected by DNA fragmentation, and the data-analysis is straight forward. Importantly, while breast and prostate tumors are typically heterogeneous, telomere DNA content was independent of the fraction of normal cells within the tumor. For instance patient 6382 had a good outcome and high telomere DNA content, although the tumor contained 10% normal cells. The tumor from patient 640 also contained 10% normal cells however; this patient had a poor outcome and low telomere DNA content. Since our analyses were based on the median telomere DNA content, additional studies will be necessary to establish the telomere DNA contents that predict favorable and poor outcomes. Finally, prospective investigations are necessary to confirm the prognostic import of telomere DNA content in these and, potentially, other cancers.

Table 1: Summary of Breast Study Group

	N	Ethnicity[1]			Age @ DX		Lymph Nodes[4]		Estrogen Receptor[4]		Progesterone Receptor[4]		Tumor Size[5] (cm)		Tumor Grade[6]		
Characteristic		**A**	**H**	**O**	**M[2]**	**R[3]**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**< 2**	**> 2**	**1**	**2**	**3**
Initial Breast Study	28	15	10	1	48	33-75	24	2	10	6	12	5	2	16	5	9	7
Extended Breast Study	48	24	21	1	48	33-89	35	11	21	11	20	12	6	32	8	13	18

Characteristics of the breast study group are shown. 1). Patients' ethnicities were self-reported and classified as Anglo (A), Hispanic (H), or Other (O). 2). Median age. 3). Range of age of diagnoses. 4). Tumors' lymph node, estrogen-receptor and progesterone-receptor statuses. 5). Tumors were divided into two groups based on their size in largest dimension. 6). Tumors' histopathological grade.

Table 2: Summary of Prostate Study Group

	N	Ethnicity[1]			Age @ DX		Lymph Nodes[4]		Seminal Vesicles[4]		Gleason Sum Score	
Characteristic		**A**	**H**	**O**	**M[2]**	**R[3]**	**Pos**	**Neg**	**Pos**	**Neg**	**M[5]**	**R[6]**
Prostate Study Group	51	28	12	2	67	53-76	6	39	28	8	7	2-9

Characteristics of the prostate study group are shown. 1). Patients' ethnicities were self-reported and classified as Anglo (A), Hispanic (H), or Other (O). 2). Median age. 3). Range of age of diagnoses. 4). Tumors' lymph node, seminal vesicle status. 5). Median Gleason sum score 6). Range Gleason sum score.

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FIGURE LEGENDS

Figure 1: Telomere DNA Content in the Initial Breast Cancer Study

A. Breast tumors were divided into two groups, recurrent and disease-free, based on patient outcome 84 months after surgery. P value (<0.01) was calculated by Wilcoxon-Rank Sums analysis. Box plots show patients outcome on the X-axis and telomere DNA content, as a fraction of the placental control, on the y-axis.

B. Breast tumors were divided into groups, high and low, based on median telomere DNA contents (TC). TC greater than or equal to the median is defined as High TC and TC less than the median is defined as Low TC. P value (0.01) was calculated by Kaplan-Meier analysis. Plots show months of disease-free survival up to 84 months on the X-axis and surviving fraction on the y-axis.

Figure 2: Telomere DNA Content in the Extended Breast Cancer Study

The 48 human breast tumors were divided into two groups and analyzed as described in Figure 1 ($p = 0.03$).

Figure 3: Telomere DNA Content in the Prostate Cancer Study

A. Prostate tumors were divided into two groups, recurrent and disease-free, as described in Figure 1 ($p=0.01$).

B. Prostate tumors were divided into two groups and analyzed as described in Figure 1 ($p = 0.04$).

Figure 1: Telomere DNA Content Predicts Disease Recurrence and Clinical Outcome

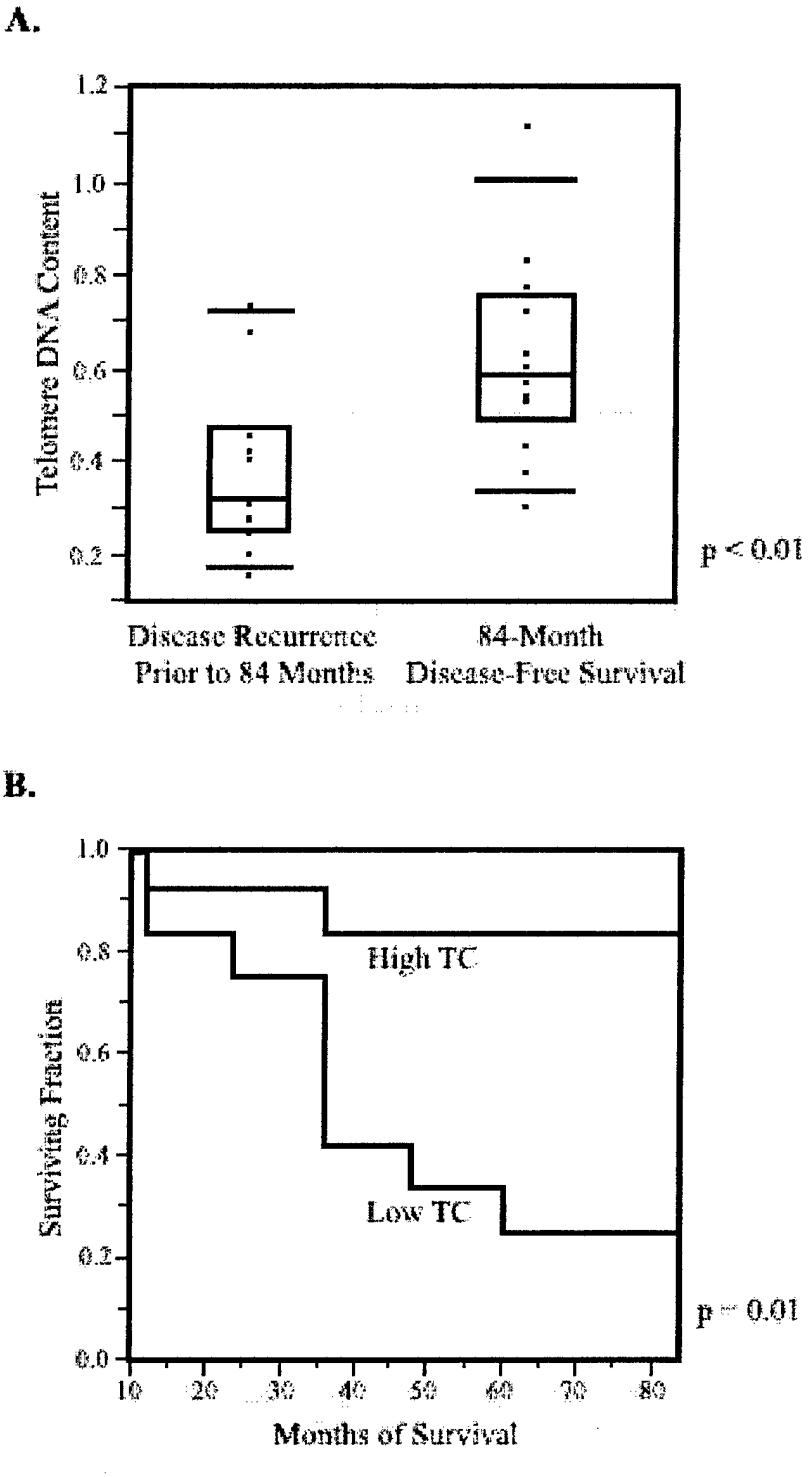

Figure 2: Telomere DNA Content Predicts Clinical Outcome in an Extended Breast Cancer Study Group

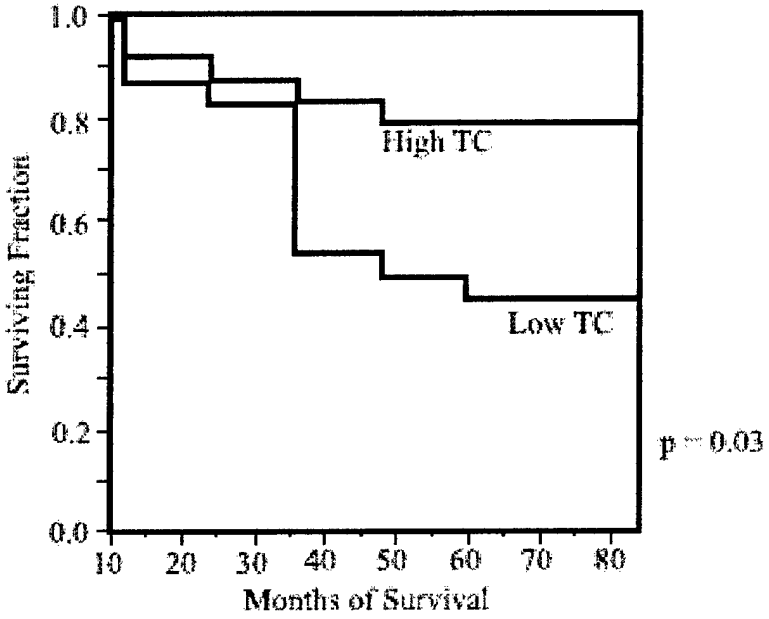

Figure 3: Telomere DNA Content Predicts Recurrence and Clinical Outcome in Prostate Cancer